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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of:	Jay Patrick SLACK
Serial No.:	10/538,038
Filed:	June 8, 2005
Examiner:	Scott LONG
Art Group:	1633
Title:	**G-PROTEINS**

MAIL STOP APPEAL BRIEF **November 15, 2010**
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

APPELLANTS' BRIEF ON APPEAL PURSUANT TO 37 CFR § 41.37

SIR:

This is an appeal from the final rejection of claims 1, 6-13, 18-26, 28-34 and 37 of the present application.

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(1) REAL PARTY IN INTEREST

The real party in interest is Givuadan SA by virtue of an assignment recorded in the United States Patent and Trademark Office on July 11, 2005, at Reel 016487, Frame 0034.

(2) RELATED APPEALS AND INTERFERENCES

There are no related appeals or interferences.

(3) STATUS OF CLAIMS

The original filed application included original claims 1-15, of which claims 2-4, 7-12 and 14-15 were amended, and claims 16 and 17 were added in a preliminary amendment dated June 8, 2005. In response to a first non-final Office Action, claims 1, 5-9 and 13 were amended, and claims 3-4 and 14-17 were cancelled in the submission dated June 16, 2007. The Examiner issued a Final Office Action on September 24, 2007, whereupon the applicants filed an after-final response on November 26, 2007, amending claims 1 and 5-8. However, the applicants were notified in the Advisory Action of December 5, 2007 that these amendments were not entered. Thereafter, the applicants filed a Request for Continued Examination (RCE) and a response amending claims 1, 2, and 6-8. Claims 18-34 were added as new, and claim 5 was cancelled. The Examiner then issued first Office Action after the RCE dated March 20, 2008. The applicants responded on May 19, 2008, but did not make any amendments to the claims. A second Final Office Action was issued, in response to which the applicants amended claims 1, 18, 21 and 24, and cancelled claims 2 and 27, and filed a second

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RCE on March 30, 2009. The Examiner then issued a non-final Office Action after the second RCE dated April 16, 2009. The applicants responded by amending claims 1, 11-13, 18, 21, 24, and 32-34, and adding claims 35-39 in their August 13, 2009 response. The Examiner issued yet another Final Office Action on November 13, 2009, and held that claims 35, 36, 38 and 39 withdrawn as being directed to an unelected invention. The applicants then filed a Notice of Appeal on May 13, 2010. Claims 1, 6-13, 18-26, 28-34 and 37 are pending and currently stand rejected. This is an appeal of the rejection of those claims.

(4) STATUS OF AMENDMENTS

In response to the Final Office Action dated November 13, 2009, the Appellants filed a Notice of Appeal. There are no unentered or pending amendments to these claims.

(5) SUMMARY OF THE CLAIMED SUBJECT MATTER

There are five independent claim, viz., claims 1, 18, 21, 24 and 37.

Claim 1 is directed to a Gα16/gust 44 or Gα 15/gust44 chimeric G-protein wherein the last 44 amino acids of the Gα16/gust 44 or Gα 15/gust44 protein sequence are replaced with a 44 amino acid unit of Gustducin, where such 44 amino acid unit of Gustducin is the last 44 amino acids of SEQ ID NO:2 where the chimeric protein, when employed in a mammalian cell-based assay increases the signal strength to at least double the signal strength of wild type Gα16. (page 4, line 21 to page 5, line 2; page 15, lines 14-20)

Claims 6-13 depend either directly or indirectly from claim 1.

Claim 18 is directed to a Gα16/gust 44 or Gα 15/gust44 chimeric G-protein wherein the last 44 amino acids of the Gα16/gust 44 or Gα 15/gust44 protein sequence are replaced with a 44 amino acid unit of Gustducin, where such 44 amino acid unit of Gustducin is the last 44 amino acids of SEQ ID NO:2, and wherein the resulting $_{Gaq\text{-}gust44}$ chimeric G-protein has a sequence homology of at least 80% in the last 44 amino acids of SEQ ID NO:2 where the chimeric protein, when employed in a mammalian cell-based assay increases the fluorescence signal strength by at least double the signal strength of wild type Gα16. (page 4, line 21 to page 5, line 2; page 5, lines 21-23; page 15, lines 14-20)

Claims 20 and 28-34 depend either directly or indirectly from claim 18.

Claim 21 is directed to Gα16/gust 44 or Gα 15/gust44 chimeric G-protein wherein the last 44 amino acids of the Gα16/gust 44 or Gα 15/gust44 protein sequence are replaced with a 44 amino acid unit of Gustducin, where such 44 amino acid unit of Gustducin is the last 44 amino acids of SEQ ID NO:2, and wherein the resulting $_{Gaq\text{-}gust44}$ chimeric G-protein has a sequence homology of at least 80% to SEQ ID NO:2. (page 4, line 21 to page 5, line 2; page 5, lines 21-23)

Claims 23 and 23 depend directly from claim 21.

Claim 24 is directed to a Gα16/gust 44 or Gα 15/gust44 chimeric G-protein wherein the last 44 amino acids of the Gα16/gust 44 or Gα 15/gust44 Go(15/gust44 protein sequence are replaced with a 44 amino acid unit of Gustducin, where such 44 amino acid unit of Gustducin is the last 44 amino acids of SEQ ID NO:2, and

wherein the resulting $G_{\alpha q\text{-}gust44}$ chimeric G-protein has a sequence homology of at least 80% to SEQ ID NO:2 and the chimeric protein binds to one or more of the human bitter, sweet and umami taste receptors. (page 4, line 17 to page 5, line 2; page 5, line 21-23)

Claims 25 and 26 depend directly from claim 24.

Finally, claim 37 is directed to a Gα16/gust 44 or Gα 15/gust44 chimeric G-protein wherein the last 44 amino acids of the Gα16/gust 44 or Gα 15/gust44_protein sequence are replaced with a 44 amino acid unit of Gustducin, where such 44 amino acid unit of Gustducin is the last 44 amino acids of SEQ ID NO:2, and where the chimeric protein binds to one or more of the human bitter, sweet and umami taste receptors. (page 4, line 17 to page 5, line 2)

(6) GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

I. The rejection of claims 1, 6-13, 18-26, 28-34 and 37 under 35 U.S.C. §103(a) as obvious over Margolskee (US 5,817,759) in view of Yao et al. (US 7,941,457) and further in view of Ruiz-Avila et al. (PNAS. July 17, 2001. vol. 98; No. 15: 8868-8873).

(7) ARGUMENT

I. REJECTION OF CLAIMS 1, 6-13, 18-26, 28-34 and 37 UNDER 35 U.S.C. §103(a) AS OBVIOUS OVER MARGOLSKEE (US 5,817,.759) IN VIEW OF YAO ET AL. (US 7,941,457) AND FURTHER IN VIEW OF RUIZ-AVILA ET AL. (PNAS. July 17, 2001. vol. 98; No. 15: 8868-8873).

The appellants respectfully submit that the Examiner erred in rejecting claims under 35 U.S.C. §103(a) as obvious over Margolskee (US 5,817,759) in view of Yao et al. (US 7,941,457) and further in view of Ruiz-Avila et al. (PNAS. July 17, 2001. vol. 98; No. 15).

A. A *prima facie* case of obviousness cannot be established against the presently claimed invention

The Examiner stated that Margolskee and Ruiz-Avila teach that the carboxy terminius is important to the function of sensation-specific G-proteins. The Examiner then cites to Yao as further teach chimeric Gq variants and the isolated nucleic acids encoding the same. In one embodiment, the chimeric Gq protein variants comprise C-terminal sequences from transducin or G_{aqir}. With respect to homology, the Examiner noted that Margolskee teaches that the α subunits of gustducin and the transducins comprise a subfamily of closely related proteins, and that Ruiz suggests a strong homology between gustducin and the transducins, and the importance of the C-terminus. The Examiner readily acknowledges that no single reference couples gustducin to Gq protein variant, and is therefore left to rely on Yao to teach chimeric Gq protein variants comprising C-terminal sequences from transducin having up to 44 amino acids of the C-terminus of transducin.

1. A skilled artisan would not have reasonably expected that the presently claimed invention would be successful

Prior art references can be modified or combined to reject claims as *prima facie* obvious only if there is a reasonable expectation that the modification or combination

would have been successful. *In re Merck & Co., Inc.*, 800 F.2d 1091, 231 USPQ 275 (Fed. Cir. 1986). Moreover, evidence showing that there was no expectation of success may support a conclusion that the claims are not obvious. *In re Rinehart*, 531 F.2d 1048, 189 USPQ 143 (CCPA 1976). The appellants submit that a *prima facie* case of obvious cannot be established against the presently claimed invention because at the time of the invention, a skilled artisan could not have reasonably expected that the claimed chimeric protein would be possible because the art is inherently unpredictable, and the appellants have demonstrated the lack of replicable functionality when even slight changes are made to the constituents of the chimeric protein.

The appellants submit that no predictions are possible regarding whether specific chimeric embodiments are functional, [i.e., whether they would be promiscuous and transmit a signal to the receptor strong enough to be useful in a screening method for the embodiments claimed and for even the more similar one that are not claimed.

To work in a screening method, binding and/or signal transduction activity are both necessary, but not sufficient prerequisites. Changes in the constituents of the chimera may affect the three dimensional shape and other required functionalities, including, in particular, promiscuity, which the present invention successfully increases. An increase in promiscuity of a given chimeric protein is at least as unpredictable as its general functionality. The same principle also applies to signal strength.

To illustrate, Yao fails to demonstrate promiscuity even for the mouse Gαq-protein variants disclosed(MGq(DeltaN-HVD-HA)-t5 and MGq(DeltaN-HVD-HA-t44). The only functional test in Yao is one using a mouse bitter receptor (MT2R5). It is therefore unclear from Yao whether the receptors will also work for sweet/umami.

While the appellants acknowledge that there are homologies suggested in the prior art, the appellants note that with biological entities, there is a great deal of uncertainty, even with a high degree of homology. Yao's mouse Gαq protein does not necessarily predict that increased promiscuity or signal strength for human Gαq, or other Gαq class proteins as claimed.

As support, the appellants submit to the Board an additional reference that discusses the art recognized level of unpredictability: Ueda et al. 2003, The Journal of Neuroscience 23(19): 7376-7380, published after the filing date of the present invention. This publication is directed various chimeric protein variants employing the Gq-class protein G_{16}: $G_{16gust5}$; $G_{16gust11}$; $G_{16gust23}$; $G_{16/gust37}$; and $G_{16/gust44}$.

Ueda tested these domains *not* to increase signal strength in a screen, but to determine specific domains of gustducin that are necessary for T2R coupling, with a view to determining the molecular basis for the interaction, which is virtually unknown, according to Ueda.

Taking into account Ruiz-Avila, if the art indeed had a minimum level of predictability, as advanced by the Examiner, the $G_{16gust5}$ variant would be expected to work, and to work similarly to the variants comprising a longer part of the gusducin C-terminus. However, this is not the case. As reported in Ueda, the G16gust5 variant

does not work, or at least differs significantly from how the G16gust44 variant works, as set forth in Ueda's abstract:

> Bitter taste reception is a conserved chemical sense against the ingestion of poisonous substances in mammals. A multigene family of G-protein-coupled receptors, T2R (so-called TAS2R or TRB) receptors and a G-protein subunit (Gα), gusducin, are believed to be key molecules for its perception, but little is known about the molecular basis for its interaction. Here, we use a heterologous expression system to determine a specific domain of gustducin necessary for the T2R coupling. Two chimeric Ga16 proteins harboring 37 and 44 gustducin-specific sequences at their C termini ($G_{16/gust37}$ and $G_{16gust44}$) responded to different T2R receptors with known ligands, but $G_{16/gust23}$, $G_{16/gust11}$, and $G_{16/gust5}$ did not. (Ueda, Abstract, lines 1-6).

Similarly, the G16/gust11 variant and the supposedly equally predicatable G16/gust23 variant also do not work as well as the G16/gust44 does. According to Ueda, both showed no signals while $G_{16/gust44}$ showed high signals.

The examiner argues that Ueda et al. does not stand for the proposition originally advanced by applicant - that Ueda et al. fully contradicts the examiner's position in that some of Ueda's constructs do work.

By the same token however, some of Ueda's constructs do not work. The disclosure of Yao teaches chimeric Gq variants where, in a preferred embodiment, from 5 to 44 amino acids in the C terminus are substituted. Yao thus discloses a genus [range] where over 80% of the species in his genus are inoperable.

The Examiner's argument and basis for rejection of applicant's claims requires predictability. To succeed is establishing a *prima facie* case of obviousness, all of these variants, which have a transducin C-terminus of 5 and up to 44 amino acids as taught

by Yao, i.e. including the -t5 variant, would have to actually work giving robust signal strength in a screening method.

They did not.

On the contrary, Ueda reports that none of these variants responded to different T2R receptors with known ligands; contrary to the position taken by the examiner, neither of the chimeric proteins having shorter C-termini worked, thereby clearly demonstrating a high level of unpredictability.

The experimenter attempting to develop a functional chimera, before trying it out experimentally, i.e. without applying hindsight, would find it impossible to predict which domains will continue to bind, interact and transmit their signal with all interacting partners in their new three dimensional environment. A chimeric protein might lose or acquire new unwanted functionality leading to incompatibility with any one of its partners, either in binding, in interaction or in signal transmission.

For example, G-proteins are heterotrimeric and consist of alpha, beta and gamma subunits, so any change would be required not to significantly affect the interaction with the other subunits. Yao et al. is in agreement with this point, stressing the central part of this type of G-protein with various components in signaling:

> Intracellular signaling is mediated through various effector enzymes, including cGMP phosphodiesterase, phospholipase C, adenylate cyclase, etc. (see Kinnamon & Margolskee, 1996, Curr. Opinion Neurobiol. 6: 506 513). Most effector proteins interact with the Gα, although Gβ γ subunits also contribute to the specificity of receptor-G protein coupling (Xu et al., 1998, J. Biol. Chem. 273(42): 27275 79). (Yao, col. 1, lines 35-42)

Further, even if general functionality remained, it is even more unclear whether the chimeric protein would have the necessary signal strength for a screening method or the desired increased promiscuity, e.g. being activated and transmitting the signal of both bitter and sweet receptors.

Ueda et al. did not set out to establish a system of increased promiscuity and signal strength. Instead Ueda was researching the relative importance of the various domains, and appears rather surprised by the findings, especially by the fact that the G16/gust23 variant did not work, even though the a5 helix was believed to be the major factor:

> In contrast, G16/gust23 that contained the a5 helix of gustducin appeared not to associate, although numerous studies have attested to the importance of the α5 helix in receptor coupling. Similarly, G16/gust11 and G16/gust5 did not cause T2R activity. These results indicated that the a5 helix and extreme C terminus of gustducin were insufficient for detection of T2R activities, and the β6 sheet, in addition to the α5 and C-terminal β-sheet, is indispensable for signal transduction of T2Rs. (Ueda 7379, top of right col.)

This indispensability was not previously known, Ueda being published after the priority date of the present application.

In view of Yao, Ueda's results may at first glance seem somewhat surprising, provided one assumes a predictability that the field simply does not have. The skilled person might hope and try out variations to see which one works, but would certainly not do so with a reasonable expectation of success. Notably, Yao exemplified and specifically disclosed only the mouse Gαq-t5 and mouse Gαq-t44 variants (but not hGαq-t5 nor hGαq-t44, nor any mouse or human G16 variant, compare table I in Yao).

Further, Yao tested only the mT2R5 receptor, thereby failing to show increased promiscuity.

The examiner asserts that "regar[d]less of the overall "low 58% homology" between gustducin and transducin, the portion important for function (44 aminoacids of the carboxy terminus), there is a much greater homology."

However, the C-terminus, or any component, cannot be considered in isolation, and a high homology is not necessarily an indicator of predictability and likelihood of success.

The irrelevance of a high degree of homology and the high degree of unpredictability in the art is further demonstrated by one of the prior art documents cited by the Examiner, Ruiz-Avila, which is discussed in Ueda. A gustducin mutant that is identical but for the exchange of only one amino acid in the extreme C-terminus results in a loss of the ability to activate receptors, as shown in the discussion of Ruiz-Avila in Ueda, Ueda page 7380:

> Indeed, a gustducin mutant containing a glycine-to proline substitution at position —3 can bind to taste receptor Gβy subunits and the effector, but it cannot be activated by receptors (Ruiz-Avila et al., 2001). Therefore, the extreme C terminus may also play an important role in transduction via the gustducin, Gαt1, Gαt2, and Gαi2 of T2R taste receptors. (Ueda, left col., lines 11-16)

This highlights the significance of the difference between transducin and gustducin (6 aminoacids) in the short C-terminal stretch of only 44 aminoacids in the claimed chimeric proteins.

These are combined with different backbone, G16/G15, while Yao merely

disclosed chimeric proteinscmoprising the two mouse variants of a different specific Gαq class protein, the one that gave the class its name, Gαq: MGq(DeltaN-HVD-HA)-t5 and MGq(DeltaN-HVD-HA)-t44 (both using transducin C-terminal sequences, with the five "t5" amino acids of transducin being identical to those of gustducin).

Applying the Examiner's modular view and dismissing potential interactions of C-terminal part with backbone, -t5 chimeric proteins (MGq(DeltaN-HVD-HA)-t5 and G16/gust5) being more similar to each other than the ones currently claimed, as at least the C-terminal part is identical, the difference being "only" in the combination with a different backbone, G16-t5 aka G16-gust5 would be predicted to work.

However, even if using the Gα16-t5 (Gα16/gust5) chimeric variant identical in the "critical" C-terminal part, the results differ significantly, as Ueda shows for the Gα16-gust5 chimeric variant in comparison to the Gα16-t44 variant.

This further demonstrates that not even partial identity (t5/gust5) of a "critical" part is a good indicator of transferability, and success cannot be predicted.

Accordingly, the differing C-terminus of the presently claimed proteins (Yao's t44 C-terminus differs from the C-terminus of the claimed gust44 chimera in 6 of its 44 aminoacids, having a homology of 86.3% in the C-terminal part) is even less predictable in its interaction with the backbone, especially with the different backbone of G16/G15.

Ueda's results demonstrate that homology cannot be considered in "modules", and accordingly the total homology (which is very low, 57%, as previously explained)

may be relevant as well and has to be considered in addition to the partial homology. Receptor proteins function in the three-dimensional context with their G-protein interaction partners.

When cutting out a specific "module" and transplanting it elsewhere, regardless of a higher degree of homology (or even identity, as shown by Ueda for the G16-t5/-gust5) in the transplant, this does not mean it will work in its new surroundings, e.g. with a new G16/G15 backbone. In particular, it is not predictable whether the new variant would provide a signal strength useful for screening or increase promiscuity.

G16 or its ortholog G15, both share a low degree of homology of less than 57% similarity to Gαq, which, according to Yao, is a high divergence that should result in significant differences in efficiency and selectivity of receptor coupling:

> Protein sequence similarity between Gαq and G α15/Gαq16 is less than 57% (FIG. 1). Accordingly, such high divergence should result in significant differences in efficiency and selectivity of receptor coupling. The identification of functionally active Gq protein variants could allow for the pharmacological and genetic modulation of sensory transduction pathways. (Yao, col. 4, lines 21-27)

Notably, Yao mentions G15/G16 only in general, enumerating proteins belonging to the same class as Gαq, and in particular points out the differences to Gαq ("Protein sequence similarity ... less than 57%", "high divergence"). Even for the variants disclosed (Gαq itself), no increased promiscuity is shown, as the variants are tested with only one receptor (compare Yao's table 1).

Furthermore, Yao points out problems with Gαq class proteins, and especially G16/G15, which are not true universal adaptors. In particular, signal strength is a

problem:

> Despite their promiscuity, however, Gαq class subunits do not mediate all GPCR—effector interactions. For instance, human Gα16 and its murine counterpart Gα5 are promiscuous G proteins in that they couple to GPCRs of different G protein families (Offermanns and Simon, 1995; Negulescu et al., 1997). However, they are not true universal adapters for GPCRs in that there are at least 11 GPCRs reported to be incapable of activating G.alpha.15/G.alpha.16 (Wu et al., 1992; Arai et al., 1996; Kuang et al., 1996; Lee et al., 1998; Parmentier et al., 1998; Mody et al., 2000). Similar problems arise when using Gα15/α16 to identify ligands of ORs and T2Rs (bitter taste receptors) in that (1) calcium responses to odorants are small and quickly desensitized for ORs in Gα15/α16 transiently transfected cells (Krautwurst et al., 1998); (2) most T2Rs remain orphan using cell lines stably transfected with Gαl5 (Adler et al., 2000; Chandrashekar et al., 2000); and (3) threshold concentration of denatonium measured is at least one order higher than expected for bitter receptors, hT2R4 and mT2R8 expressed in cells stably transfected with Gα15 (Adler et al., 2000; Chandrashekar et al., 2000). These problems suggest that the coupling efficiency between ORs/T2Rs and Gα15/α16 is weak and may vary within the family of ORs and T2Rs. (Yao, col. 2, lines 28-50)

> Furthermore the paralogs (G16/15 versus Gαq) are not very conserved,

suggesting distinct functions according to Yao, even though some activities are similar:

> Signaling specificity among α subunits of the same class having similar biochemical functions is not well understood in vivo. For instance, the Gαq (Gq) class includes four proteins expressed in mammals, called Gαq, Gα11, Gαq14, and Gαq15 (in mice, Gα16 in humans). Whereas orthologs of these subunits are highly conserved across species (99, 97, 96 and 85% identity, respectively), paralogs of these subunits (expressed in the same species) are not as conserved. This suggests that each type of subunit in the Gq class has a distinct function, however, when transfected into Sf9 cells, the subunits stimulated phospholipase C with similar potency and showed similar activities (Nakamura et al., 1995, J. Biol. Chem. 270: 6246 6253). Xu and colleagues subsequently showed by gene knockouts in mice that Gq.sub.60 subunits promiscuously couple to several different receptors in various cell types (1998, J. Biol. Chem. 273(42): 27275).(col. 1, line 56 to col. 2, line 4)

> This would appear to discourage the skilled artisan from replacing Gαq with

G16/G15, or at least indicate unpredictability expecting different results, in particular if the C-terminal module is replaced as well.

In any case, it remains that the result is unpredictable, as shown by Ueda's G16gust5 variant which does not appear to work, and certainly works differently from G16gust44 which Ueda tested in parallel.

Based on the foregoing, the appellants submit that at the time of the invention, a skilled artisan would not have reasonably expected that the claimed invention would be successful, and therefore respectfully request that the Honorable Board overturn the Examiner's rejection.

2. The differences between the present invention and the prior art references are outside the scope of the ordinary level of skill in the art

The presently claimed invention is directed to a $G_{\alpha q\text{-Gustducin}}$ chimeric G-protein wherein the last 44 amino acids of the $G_{\alpha q}$ protein sequence are replaced with a 44 amino acid unit of Gustducin, G15/gust 44 and G16/gust44. These chimera provide increased signal strength. Furthermore, the claimed compositions up-regulate signal strength, a property not exhibited by the prior art compositions or recognized by the prior art, thus making the claimed compositions substantially more useful for their intended purpose and providing a surprising advantage over the existing art.

Margolskee teaches that Gustducin a subunit variants may comprise polypeptide analogs wherein one or more of the specified amino acids is deleted or replaced or

wherein one, or more nonspecified amino acids are added. Margolskee also teach that the transducins comprise a subfamily of closely related proteins and that the carboxy terminal 60 amino acids of all three proteins gustducin and rod and cone transducins are highly conserved, while the carboxyl terminal 38 amino acids are identical and that the carboxyl terminal identity is of particular importance because it encompasses the site that has been implicated in G protein/receptor interactions.

In addition, Margolskee teaches SEQ ID NO:3 which consists of the last 40 amino acids of Gustducin α subunit and is 100% identical to the last 40 of SEQ ID NO:2 of the instant application.

The criticality or not of the last 44 amino acids of the G protein is at best an invitation to experiment with the 44 amino acid long chain at the end of the much larger g protein. An invitation to modify one or more of 44 amino acids is an invitation to test one or more of the literally millions, if not billions, of combinations and permutations possible when dealing with a chain of 44 individual amino acids, each of which can be individually modified. The image of searching for a needle in a haystack is immediately brought to mind.

Assuming, *arguendo*, that an intrepid experimenter would begin this apparently unrewarding task, where would he start.

The intrepid experimenter may start out, as did the examiner, with Margolskee who teaches the α subunit of a novel taste receptor cell specific G protein, gustducin, or fragments and variants of the a subunit and subtypes of G-proteins, Gal5 and Ga16.

So, the examiner argues, a hypothetical experimenter is going to focus on the α subunit and the last the 40 amino acids at the carboxy end. But Margolskee do not teach chimeric G-proteins comprising the carboxy 40 amino acids of Gustducin α subunit.

Following the examiner's journey, and with knowledge of Ruiz-Avila et al. who teach several biochemical studies suggesting that the interaction of gustducin with its cognate taste receptors is similar to that of transducin with rhodopsin and that a key result of these studies is that the C terminus of a-gustducin is a critical determinant for its interaction with taste receptors, the next step our experimenter takes is to consider Yao et al.

Yao et al. teach chimeric Gq variants and the isolated nucleic acids encoding the same.

In one embodiment, the chimeric Gq protein variants comprise C-terminal sequences from transducin or G_{odf}. Yao et al. teach our experimenter that, in a preferred embodiment, at least about five amino acids in the C terminus of the Gq protein are replaced by at least about five amino acids from the C terminus of Ga_{olf} or transducin and that up to 44 amino acids of the C terminus of transducin or G_{odf} may be incorporated.

Yao et al. indicates that the C-terminus of Gα proteins can be modified to promote promiscuity of taste receptors. Yao et al. also describe the shared homologies of Gα subunits. Further, Yao et al. also suggest that the gustducin-coupled bitter

receptor can be modified to increase promiscuity with regard to GPCR coupling.

Yao et al. report their results in Table 1. Specifically, Yao et al. show that one specific chimeric G-protein MGq(ΔN–HVD–HA)-t44 resulted in bitter taste receptor functionality as did 2 t5 chimeras MGq(ΔN-HVD-HA)-t5 and MGq (HVD-HA)-t5.

Thirteen (13) other variants show little or no functionality. An experimenter extrapolating from Yao et al.'s results would not be pointed to any specific carboxy end change since the -olf5 version didn't work. He would not be sure of the ΔN portion since it worked twice, had some functionality once and didn't work twice. The HA portion worked 3 times but didn't work 4 times. The HVD portion would appear to be the best bet.

The pertinent part of Yao et al.'s disclosure is that a tail of t5, or t44 combined with a specific construct would work but a tail of olf5 wouldn't work.

Notably, the present claims are directed to a combination with G_{15}/G_{16}, which from Yao is known to give poor signal strength.

> Similar problems arise when using $G_{\alpha 15/\alpha 16}$ to identify ligands of ORs and T2Rs (bitter taste receptors) in that (1) calcium responses to odorants are small and quickly desensitized for ORs in $G_{\alpha 15/\alpha 16}$ transiently transfected cells (Krautwurst et al., 1998); (2) most T2Rs remain orphan using cell lines stably transfected with Gα15 (Adler et al., 2000; Chandrashekar et al., 2000); and (3) threshold concentration of denatonium measured is at least one order higher than expected for bitter receptors, hT2R4 and mT2R8 expressed in cells stably transfected with Ga15 (Adler et al., 2000; Chandrashekar et al., 2000). These problems suggest that the coupling efficiency between ORs/T2Rs and $G_{\alpha 15/\alpha 16}$ is weak and may vary within the family of ORs and T2Rs.(Yao, col. 2, lines 37-50)

The conclusion drawn by the examiner is that, oblivious to this disclosure, our

experimenter would proceed, without untold numbers of experiments rising to the level of non-obviousness, to substitute the specific 44 amino acid sequence tail disclosed by applicant.

But this would require a leap that is logically blocked by Ueda et al. which discloses to our experimenter that results obtained utilizing transducin do NOT apply to gustducin.

For all the general statements about the equivalence of or interchangeability between transducin and gustducin, the actual experimental results, as reported by Ueda et al., found that transducin 5, 11 and 23 amino acid tails of transducin were not equivalent to, nor functional as, the 5, 11 and 23 amino acid tails of gustducin.

Thus, although substitutions of 5 amino acids or more is functional when the tail is transducin, over 80% of the time, Yao et al.'s chimera's are not functional as applicant's chimera even if the leap were made from transducin to gustducin.

The documentary evidence in the record of this application thus refutes the examiner's *post hoc* argument that the disclosure of a tranducin tail renders obvious a gustducin tail.

So even if our experimenter stumbled upon the idea of substituting a different tail comprising gustducin onto his molecule he would be disappointed because his chimera would not be functional until he reached 37 or preferably applicant's claimed 44 amino acid long chain.

Thus, even after this supposed series of multiple serendipitous discoveries, our

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experimenter would still not reach his goal of reproducing applicant's claimed invention.

This clearly demonstrates that, based on documentary evidence and not examiner or attorney argument, the combination of Margolskee, Ruiz-Aveda and Yao et al. do not sufficiently provide guidelines to an experimenter skilled in the art to render obvious applicant's claims and that a *prima facie* case of obviousness has not been established.

Based on the forgoing remarks, the appellants respectfully request that the Honorable Board overturn the Examiner's rejection.

(8) CONCLUSION

In view of the foregoing, Appellants respectfully request that the Honorable Board reverse the final rejection.

CONDITIONAL PETITION FOR EXTENSION OF TIME

If entry and consideration of the above requires an extension of time, Applicant respectfully requests that this be considered a petition therefor. The Commissioner is authorized to charge any fee(s) due in this connection to Deposit Account No. 14-1263.

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ADDITIONAL FEE

Applicant notes that Extension of Time Fees for months 1-3 were previously charged in this matter. Please charge only the remaining 4th month Extension of Time fee in association with this Appeal Brief. Additionally, please charge any insufficiency of fees, or credit any excess, to Deposit Account No. 14-1263.

Respectfully submitted,

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(9) CLAIMS APPENDIX

1. (Previously Presented) A Gα16/gust 44 or Gα 15/gust44 chimeric G-protein wherein the last 44 amino acids of the Gα16/gust 44 or Gα 15/gust44 protein sequence are replaced with a 44 amino acid unit of Gustducin, where such 44 amino acid unit of Gustducin is the last 44 amino acids of SEQ ID NO:2 where the chimeric protein, when employed in a mammalian cell-based assay increases the signal strength to at least double the signal strength of wild type Gα16.

2-5. Cancelled

6. (Previously Presented) A G-protein according to claim 1 encoded for by the nucleic acid set forth in SEQ ID NO:1.

7. (Previously Presented) A nucleic acid comprising the nucleotide sequence set forth in SEQ ID NO:1 encoding for a G-protein according to claim 1.

8. (Previously Presented) An expression vector comprising nucleic acid comprising the nucleotide sequence set forth in SEQ ID NO:1 encoding for a G-protein according to claim 1.

9. (Previously Presented) A host cell transformed with an expression vector according to claim 8.

10. (Previously Presented) A method of producing a chimeric G-protein according to claim 1 comprising the step of culturing host cells having contained therein an expression vector encoding for the chimeric G-protein, under

conditions sufficient for expression of said G-protein, thereby causing production of the protein, and recovering the protein produced by the cell.

11. (Previously Presented) The method of analysis and discovery of modulators of taste receptors of claim 12 where the taste receptors are bitter receptors.

12. (Previously Presented) A method of analysis and discovery of modulators of taste receptors employing a mammalian cell-based assay employing a transfected gene or cDNA encoding a chimeric protein of claim 1 and a taste receptor, the method comprising the steps of contacting a compound with cells, and determining the functional effect of the compound on the chimeric G-protein.

13. (Previously Presented) A method according to claim 10 wherein expression of said G-protein is measured by signal transduction output determined by measuring the changes in intracellular messengers IP3 or calcium^{2+}.

14-17. Cancelled

18. (Previously Presented) A Gα16/gust 44 or Gα 15/gust44 chimeric G-protein wherein the last 44 amino acids of the Gα16/gust 44 or Gα 15/gust44 protein sequence are replaced with a 44 amino acid unit of Gustducin, where such 44 amino acid unit of Gustducin is the last 44 amino acids of SEQ ID NO:2, and wherein the resulting $_{Gaq\text{-}gust44}$ chimeric G-protein has a sequence homology of at least 80% in the last 44 amino acids of SEQ ID NO:2 where the chimeric protein, when employed in a mammalian cell-based assay increases the fluorescence signal strength by at least double the signal strength of wild type Gα16.

19. (Previously Presented) The chimeric G-protein of claim 18 having a

sequence homology of at least 90% in the last 44 amino acids of SEQ ID NO:2.

20. (Previously Presented) The chimeric G-protein of claim 18 having a sequence homology of at least 95% in the last 44 amino acids of SEQ ID NO:2.

21. (Previously Presented) A Gα16/gust 44 or Gα 15/gust44 chimeric G-protein wherein the last 44 amino acids of the Gα16/gust 44 or Gα 15/gust44 protein sequence are replaced with a 44 amino acid unit of Gustducin, where such 44 amino acid unit of Gustducin is the last 44 amino acids of SEQ ID NO:2, and wherein the resulting $_{Gaq\text{-}gust44}$ chimeric G-protein has a sequence homology of at least 80% to SEQ ID NO:2.

22. (Previously Presented) The chimeric G-protein of claim 21 having a sequence homology of at least 90% to SEQ ID NO:2.

23. (Previously Presented) The chimeric G-protein of claim 21 having a sequence homology of at least 95% to SEQ ID NO:2.

24. (Previously Presented) A Gα16/gust 44 or Gα 15/gust44 chimeric G-protein wherein the last 44 amino acids of the Gα16/gust 44 or Gα 15/gust44 Go(15/gust44 protein sequence are replaced with a 44 amino acid unit of Gustducin, where such 44 amino acid unit of Gustducin is the last 44 amino acids of SEQ ID NO:2, and wherein the resulting $_{Gaq\text{-}gust44}$ chimeric G-protein has a sequence homology of at least 80% to SEQ ID NO:2 and the chimeric protein binds to one or more of the human bitter, sweet and umami taste receptors.

25. (Previously Presented) The chimeric G-protein of claim 24 having a sequence homology of at least 90% to SEQ ID NO:2.

26. (Previously Presented) The chimeric G-protein of claim 24 having a sequence homology of at least 95% to SEQ ID NO:2.

27. Canceled

28. (Previously Presented) A nucleic acid encoding for a G-protein according to claim 18.

29. (Previously Presented) An expression vector comprising nucleic acid comprising the nucleotide sequence encoding for a G-protein according to claim 18.

30. (Previously Presented) A host cell transformed with an expression vector according to claim 29.

31. (Previously Presented) A method of producing a chimeric G-protein according to claim 18 comprising the step of culturing host cells having contained therein an expression vector encoding for the chimeric G-protein, under conditions sufficient for expression of said G-protein, thereby causing production of the protein, and recovering the protein produced by the cell.

32. (Previously Presented) [[A]] The method of claim 33 where the taste receptor is a bitter taste receptor.

33. (Previously Presented) A method of analysis and discovery of modulators of taste receptors using the chimeric proteins of claim 18 employing a mammalian cell-based assay employing a transfected gene or cDNA encoding a chimeric Gα16/gust 44 or Gα 15/gust44 and a taste receptor, the method comprising the steps of contacting a compound with cells, and determining the

functional effect of the compound on chimeric G-protein.

34. (Previously Presented) A method according to claim 31 wherein expression of said G-protein is measured by signal transduction output determined by measuring the changes in intracellular messengers IP3 or calcium^{2+}.

35. (Withdrawn) The method of claim 33 where the taste receptor is a sweet taste receptor.

36. (Withdrawn) The method of claim 33 where the taste receptor is a umami taste receptor.

37. (Previously Presented)A Gα16/gust 44 or Gα 15/gust44 chimeric G-protein wherein the last 44 amino acids of the Gα16/gust 44 or Gα 15/gust44 protein sequence are replaced with a 44 amino acid unit of Gustducin, where such 44 amino acid unit of Gustducin is the last 44 amino acids of SEQ ID NO:2, and where the chimeric protein binds to one or more of the human bitter, sweet and umami taste receptors.

38. (Withdrawn) The method of claim 12 where the receptors are sweet receptors.

39. (Withdrawn) The method of claim 12 where the receptors are umami receptors.

(10) EVIDENCE APPENDIX

1. Ueda et al., *Journal of Neuroscience*, Aug. 13, 2003; 23(19): 7376-7380

Cellular/Molecular

Functional Interaction between T2R Taste Receptors and G-Protein α Subunits Expressed in Taste Receptor Cells

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Bitter taste perception is a conserved chemical sense against the ingestion of poisonous substances in mammals. A multigene family of G-protein-coupled receptors, T2R (so-called TAS2R or TRB) receptors and a G-protein α subunit (Gα), gustducin, are believed to be key molecules for its perception, but little is known about the molecular basis for its interaction. Here, we use a heterologous expression system to determine a specific domain of gustducin necessary for T2R coupling. Two chimeric Gα16 proteins harboring 37 and 44 gustducin-specific sequences at their C termini (G16/gust37 and G16/gust44) responded to different T2R receptors with known ligands, but G16/gust 23, G16/gust11, and G16/gust5 did not. The former two chimeras contained a predicted β6 sheet, an α5 helix, and an extreme C terminus of gustducin, and all the domains were indispensable to the expression of T2R activity. We also expressed G16 protein chimeras with the corresponding domain from other Gαi proteins, cone-transducin (Gαt2), Gαi2, and Gαz (G16/t2, G16/i2, and G16/z). As a result, G16/t2 and G16/i2 produced specific responses of T2Rs, but G16/z did not. Because Gαt2 and Gαi2 are expressed in the taste receptor cells, these G-protein αi subunits may also be involved in bitter taste perception via T2R receptors. The present Gα16-based chimeras could be useful tools to analyze the functions of many orphan G-protein-coupled taste receptors.

Key words: bitter taste; T2R receptor; G-protein α subunit; gustducin; Gα chimera; calcium imaging

Introduction

Taste perception is initially mediated by multiple signaling pathways in the taste receptor cells (TRCs) within taste buds in the oral epithelium. Bitter taste, as well as sweet taste, is believed to be detected by G-protein-coupled receptors (GPCRs), and the signaling pathways of TRCs have been the subject of intense speculation (for review, see Gilbertson et al., 2000; Lindemann, 2001; Margolskee, 2002; Montmayeur and Matsunami, 2002). α-Gustducin is a transducin-like Gαi protein selectively expressed in 20–30% of TRCs (McLaughlin et al., 1992). *In vitro* biochemical assays and *in vivo* physiological studies using knock-out mice have demonstrated that gustducin plays a key role in TRC responses to numerous bitter compounds (Wong et al., 1996). However, gustducin knock-out mice still retained substantial sensitivity to bitter compounds in physiological and behavioral assays (Wong et al., 1996; He et al., 2002). In contrast, the second family of taste receptors identified, T2R (so-called TAS2R or TRB), is a large GPCR multigene family of ~30 members in humans and rodents (Adler et al., 2000; Matsunami et al., 2000). The genes map to regions of human and mouse chromosomes implicated genetically in sensitivity to various bitter compounds and are coexpressed with gustducin, suggesting that T2R receptors could function as gustducin-linked bitter taste receptors. Currently, there are two T2R receptors that display ligand responses with an affinity range compatible with behavioral sensitivity: mouse T2R5 (mT2R5) for cycloheximide (Chandrashekar et al., 2000) and human T2R16 (hT2R16) for salicin (Bufe et al., 2002). However, the other T2R receptors remain orphan receptors with no known ligands.

To measure T2R activity, previous studies used a heterologous expression system using human embryonic kidney 293/Gα15 (HEK293/Gα15) cells (Chandrashekar et al., 2000; Bufe et al., 2002). These cells stably express the α subunit of the mouse G-protein α subunit (Gα) protein Gα15, which is thought to indiscriminately couple to many GPCRs (Offermanns and Simon, 1995). In this strategy, transfection of Gα15 into the cell system potentially allows measurements of elevated levels of intracellular calcium ($[Ca^{2+}]i$), giving a simple readout for agonist activation, although there is evidence that bitter taste transduction is mediated by Gαi-coupled receptors. However, Gα15 cannot be considered as a true universal adapter for GPCRs, because ~18% of the total number of Gαi-coupled GPCRs examined to date cannot activate its human ortholog Gα16 (Mody et al., 2000; Kostenis, 2001). Moreover, T2R receptors are believed to couple with gustducin in the native TRCs. In the present study, we constructed a variety of chimeric Gα proteins to determine the specific domain of gustducin necessary for T2R coupling and demonstrated that a specific domain of gustducin is indispensable to ligand responses compatible with behavioral sensitivity.

Materials and Methods

Materials. Animals were obtained from Shizuoka Laboratory Animal Center (Shizuoka, Japan). The human leukemic cell line, HL60, was

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obtained from the Japan Collection Research Bioresources Cell Bank (Japan). Reagents for reverse transcription PCR (RT-PCR) were obtained from Invitrogen (Carlsbad, CA) and Applied Biosystems (Branchburg, NJ). Cycloheximide was purchased from Biomol Research Laboratories (Plymouth Meeting, PA). Salicin, serum, culture media, and anti-FLAG M2 and anti-opsin (Clone RET-P1) monoclonal antibodies were from Sigma (St. Louis, MO) unless otherwise noted.

Construction of Gα proteins and chimeras. A variety of Gα subunits were obtained from a human cell line and rat tissues by RT-PCR. Human Gα16 was obtained from HL60 cells. Rat α-gustducin and Gαi2 cDNAs were from rat lingual tissues containing circumvallate papillae. Similarly, rat Gαt2 and Gαz cDNAs were obtained from the retina and brain, respectively. All of the chimeras were constructed by PCRs using human Gα16 and rat-appropriated Gα cDNAs as templates. We first constructed a series of Gα16/gustducin (G16/gust) chimeras by incorporating different lengths of gustducin amino acid sequences at the C terminus of Gα16: G16/gust44, G16/gust37, G16/gust23, G16/gust11, and G16/gust5. In addition, we also constructed Gα16-based chimeras by replacing 44 amino acid sequences at the C terminus of Gα16 with those of Gαt2, Gαz, or Gαi2 (G16/t2, G16/z, and G16/i2). All full-length α-subunit cDNAs were subcloned into a pcDNA3.1(+) mammalian expression vector (Invitrogen). The G16/gust chimeras were also tagged by a FLAG epitope and cloned into the vector for expression assay and Western blot analysis.

Construction of T2R receptors. Mouse T2R5 and human T2R16 were amplified from mouse and human genomic DNAs, respectively. We subcloned its open reading frame into pME18S-FL3 containing the first 39 amino acids of bovine rhodopsin in the frame. The sequences allow immunohistochemical detection and facilitate expression of the recombinant chemosensory receptors on the cell surface.

Transfection of HEK293T cells. HEK293T cells were cultured with DMEM and supplemented with 10% FCS (v/v) at 37°C in humidified air with 5% CO_2. For transfection, cells were seeded onto 100 mm dishes or uncoated glass coverslips in 35 mm chambers. After 24 hr at 37°C, cells were washed in DMEM medium and transfected with Gα and T2R using LipofectAmine 2000 reagent (Invitrogen). The transfection efficiencies were estimated by cotransfection of a GFP reporter plasmid or by immunohistochemistry and were typically >70%.

Western blot analysis. For Western blot analysis, a series of FLAG-tagged G16/gust chimeras was used. Cells were grown on 100 mm dishes to 70–80% confluence. Transfection was performed on 35 mm dishes with proper adjustments for the volumes and amounts of the reagents used. After 36 hr in normal growth conditions, cells were spun down briefly, resuspended in a lysis buffer (20 mM Tri-HCl, pH 7.4, 0.1% SDS, 1% Triton X-100, 1% sodium decoxycholate) containing one protease inhibitor cocktail tablet (complete mini; Roche Products, Mannheim, Germany), lysed by one cycle of freeze-thawing followed by 10 passages through a 27-gauge needle at 4°C, and centrifuged at 15,000 rpm for 30 min. Collected supernatants were used, and the protein concentrations were determined using a Bio-Rad (Hercules, CA) protein assay kit. Next, 50 μg of each protein sample was resolved on a 10% SDS-polyacrylamide gel and transferred to Immobilon-P transfer membrane (Millipore, Bedford, MA) via electroblotting. FLAG-tagged Gα chimeras were detected by an anti-FLAG antibody followed by an alkaline phosphatase-labeled anti-mouse IgG secondary antibody and then visualized by a phosphatase reaction using nitro blue tetrazolium chloride and 5-bromo-4-chlor-indolyl-phosphate (Roche Products).

Calcium imaging. We used a cell-based reporter system to examine T2R-Gα interaction (Chandrashekar et al., 2000; Bufe et al., 2002). In this system, receptor activation leads to increases in intracellular calcium $[Ca^{2+}]i$, which can be monitored at the single-cell level using the fura-2 AM calcium indicator dye. HEK293T cells were transiently transfected with a rhodopsin-tagged T2R receptor with a Gα16- or Gα16-based Gα chimera using LipofectAmine 2000 reagent. After 24–30 hr, transfected cells were loaded with 5 μM fura-2 AM for 30 min at room temperature. The loading solution was washed out, and cells were incubated in 500 μl of assay buffer (10 mM HEPES, 130 mM NaCl, 10 mM glucose, 5 mM KCl, 2 mM $CaCl_2$, and 1.2 mM $MgCl_2$, pH 7.4) and stimulated with tastants using a bath perfusion system at a flow rate of 5 ml/min. We recorded

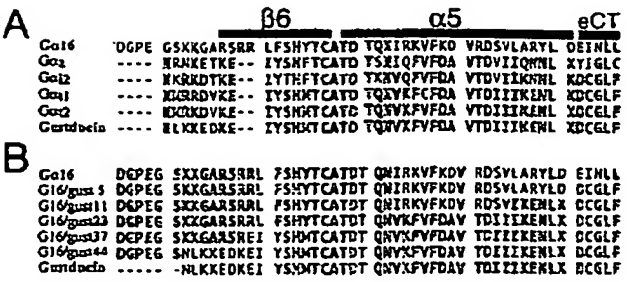

Figure 1. *A*, *B*, Alignments of C-terminal amino acid sequences of Gα16, Gαz, Gαi2, Gαt1, Gαt2, and α-gustducin (*A*) and a variety of G16/gust chimeras (*B*). Conserved sequences are in gray, and the sequences that differ from the majority are in black. Putative secondary structures on the basis of the Gαt1 crystal structure are indicated by shaded bars above the Gα16 sequence in *A*. eCT, Extreme C terminus.

$[Ca^{2+}]i$ changes using an Olympus IX-70 (Olympus Optical, Tokyo, Japan) equipped with the ARGUS–HiSCA system (Hamamatsu, Shizouka, Japan). Aquisition and analysis of the fluorescence images were done using ARGUS–HiSCA version 1.65 software. Generally, $[Ca^{2+}]i$ response was measured by sequentially illuminating cells at 340 and 380 nm and monitoring the fluorescence emission at 510 nm using a cooled CCD camera. At the beginning of each experiment, 10 μM isoproterenol was applied for 10 sec. A 180 sec interval was then left between each tastant application to ensure that cells were not desensitized as a result of the previous application of tastants. In all cases, we measured the entire camera field. As a control, we used isoproterenol (10 μM) to stimulate endogenous β-adrenergic receptors, proving that the Gα16-dependent signal transduction cascade was functional. Approximately 70–80% of all cells in the camera field responded to isoproterenol, whereas ~15–20% of all cells in the field showed dose-dependent responses to agonists in the transient transfection experiments. The proportion of responders was about half of that found by immunohistochemistry, which was similar to that in a previous study using HEK/Gα15 cells (Bufe et al., 2002).

Results

Figure 1*A* shows the alignment of the C-terminal amino acid sequences of the Gα used. Murine Gα15 has been successfully used to determine the function of two T2R receptors when stably expressed in HEK293 cells. Because its human ortholog, Gα16, is also known to interact with a wide variety of GPCRs, we first examined whether Gα16 could couple to T2R receptors in our transient expression system. Although we expressed Gα16 with mT2R5, a cycloheximide receptor in the mouse, by transient transfection in HEK293T cells, the T2R receptor did not respond to the cycloheximide (Fig. 2). Similarly, hT2R16, which has been reported to react with a plant bitter tastant salicin, failed to respond to it (Fig. 2). In both cases, 10 μM isoproterenol increased $[Ca^{2+}]i$ by activating an endogenous β-adrenergic receptor present in HEK293T cells. Untreated cells and cells without Gα16 did not respond to isoproterenol, indicating that human Gα16 could mediate intracellular calcium mobilization but could not couple to T2R receptors in the present assay system. We thus used Gα16 as the basis of Gα chimeras for a functional assay on the basis of calcium imaging.

As numerous studies on Gαi subunits have attested to the importance of the C-terminal tail of the α subunit as one of the major receptor contact regions, we constructed a series of Gα16/gustducin chimeras by incorporating different lengths of gustducin sequences at the C terminus of Gα16 (Fig. 1*B*). First, because the α5 helix is a known contact region for receptors (Lichtarge et al., 1996), we replaced the entire α5 helix of Gα16 with that of

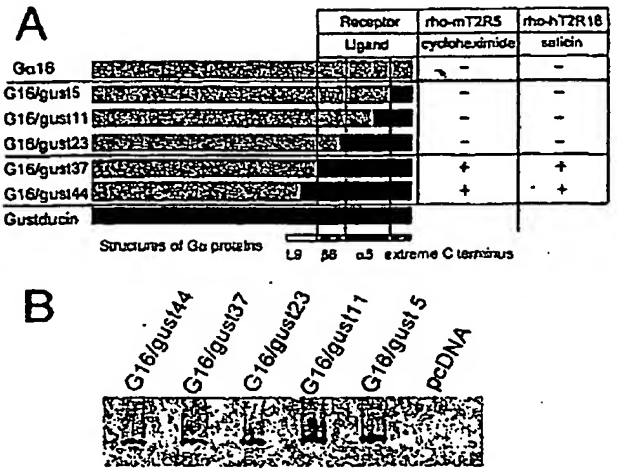

Figure 2. *A*, Schematic illustrations of chimeric G16/gust proteins with different lengths of C terminal amino acids found in gustducin and their abilities to couple to T2Rs. +, Specifically responded to the ligand in dose-dependent manner; −, did not exhibit any responses. *B*, Immunoblot analysis of FLAG-tagged chimeric G16/gust subunits expressed in HEK293T cells, stained with the anti-FLAG M2 monoclonal antibody. The FLAG epitope tag did not influence the functional activity of G16/gust chimeras.

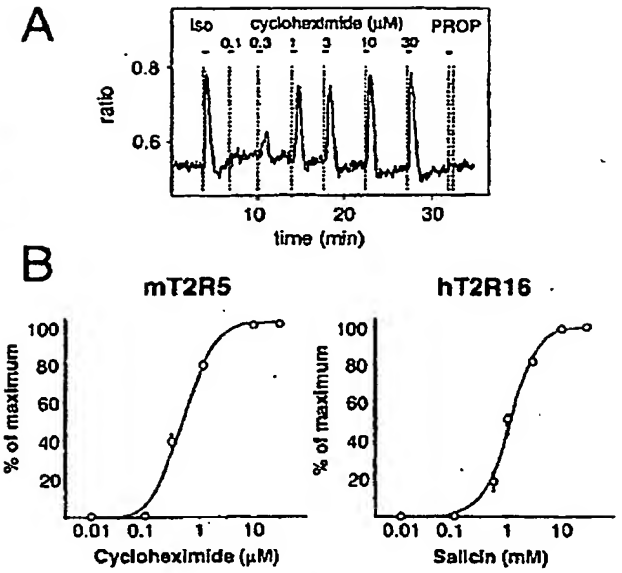

Figure 3. *A*, $[Ca^{2+}]i$ responses in HEK293T cells expressing G16/gust37 and rho-mT2R5 when treated with multiple pulses of 10 μM isoproterenol (Iso), cycloheximide, and 3 mM PROP (6-*n*-propylthiouracil). Isoproterenol was used to ascertain that the G16-dependent signaling cascade was functional. Horizontal bars above the traces indicate the time and duration of tastant pulses. Cycloheximide triggered robust receptor activation, but PROP did not. Similar results were obtained when G16/gust44 was used in place of G16/gust37. *B*, Dose-dependent curves of the effects of the ligands on the $[Ca^{2+}]i$ in cells expressing G16/gust37 and the T2R receptor indicated.

gustducin. The resultant chimera was named G16/gust23; for G16/gust23 and subsequent chimeras, the number after the latter gust indicates the number of gustducin residues present in the C terminus of the construct. Second, another region determining coupling selectivity is the extreme C terminus (also called C-terminal turn or β-turn) (Conklin et al., 1993; Blahos et al., 2001). G16/gust5 contains the minimum sequences of gustducin that correspond to the region decisive for coupling of Gα proteins with specific receptors. G16/gust11 was designed from a Gα COOH-terminal minigene vector to explore the coupling mechanisms of receptors (Gilchrist et al., 2002). Last, the region between α4 and α5 helices that includes the L9 loop and β6 sheet is also involved in coupling selectivity, probably by directly interacting with the receptors of the rhodopsin-like family (family 1 GPCRs) (Noel et al., 1993). G16/gust44 contains all the structures, an L9 loop, a β6 sheet, an α5 helix, and an extreme C terminus of gustducin, whereas G16/gust37 includes the latter three structures. We also made FLAG epitope-tagged G16/gust chimeras, but the results obtained using these epitope-tagged chimeras were identical to those using the Gα chimeras without epitope tags.

We used a well established transient expression system to examine the ability of the G16/gust chimeras to interact with T2R receptors (see Materials and Methods). We first examined the response against cycloheximide in HEK293T cells coexpressing the rhodopsin-mT2R5 (rho-mT2R5) with G16/gust44, G16/gust37, G16/gust23, G16/gust11, or G16/gust5. When transfected with T2R and either G16/gust44 or G16/gust37, cells specifically responded to cycloheximide (Figs. 2 and 3). The response was receptor- and Gα chimera-dependent, because cells lacking either of these components did not cause a [Ca2+]i increase, even at a 1000-fold higher cycloheximide concentration. In this assay, the EC_{50} value of mT2R5 was 0.5 μM, and the threshold was ~0.2 μM (Fig. 3). These responses resembled those obtained from behavioral experiments with rodents (sensitivity threshold, ~ 0.25 μM), indicating that G16/gust44 and G16/gust37 can functionally couple to mT2R5. In contrast, the other G-protein chimeras (G16/gust23, G16/gust11, and G16/gust5) failed to mediate the cycloheximide response under identical experimental conditions (Fig. 2). These G16/gust chimeras did not mediate any responses, even at 1000-fold higher ligand concentration. Hence, we checked the expression of the G16 chimeras by Western blot analysis using an anti-FLAG M2 monoclonal antibody for the immunodetection. As shown Figure 2, all chimeras were detected by the antibody in proteins prepared from HEK293T transfected with the FLAG-tagged chimeras. There were no differences in protein expression between them (Fig. 2).

The ability of G16/gust44 and G16/gust37 to interact productively with the mT2R5 receptor prompted us to further investigate their capacity to functionally associate with another T2R receptor. HEK293T cells were cotransfected with either G16/gust44 or G16/gust37 and rho-hT2R16. In transfected cells, stimulation of the ligand for hT2R16 (salicin) significantly increased $[Ca^{2+}]i$ (Fig. 2). Such $[Ca^{2+}]i$ increases were receptor- and Gα chimera-dependent and were in a dose-dependent manner. The EC_{50} and threshold were ~2 and 0.2 mM, respectively (Fig. 3). These closely resembled those obtained in experiments with human subjects reported previously (Bufe et al., 2002). Thus, G16/gust44 and G16/gust37 successfully interacted with hT2R16 in addition to mT2R5. However, the other chimeras (G16/gust23, G16/gust11, and G16/gust5) did not mediate any ligand responses via hT2R16. Thus, the C-terminal 37 amino acid residues containing the predicted β6 sheet, α5 helix, and extreme C terminus of gustducin may be necessary for productive functional expression of T2R taste receptors.

It has been suggested that in native mammalian TRCs, bitter taste may also be mediated by Gα proteins other than gustducin. We tested whether the T2Rs studied could associate with the

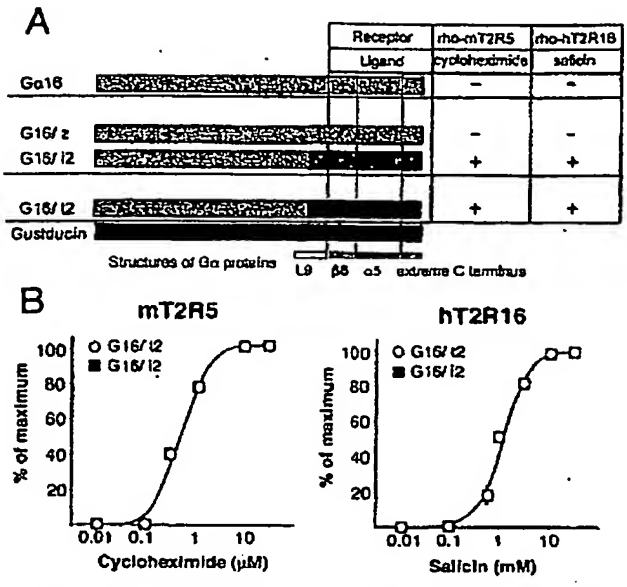

Figure 4. *A*, Schematic illustrations of chimeric G16 proteins with 44 C terminal amino acids of different Gα proteins and their ability to couple to T2Rs. +, Specifically responded to the ligand in a dose-dependent manner; −, did not exhibit any responses. *B*, Dose-dependent curves of the effects of ligands on the $[Ca^{2+}]i$ in cells expressing G16/t2 or G16/i2 and the T2R receptor indicated.

C-terminal domain of other Gα proteins corresponding to the β6 sheet, α5 helix, and extreme C terminus of gustducin. We constructed Gα16 chimeras with 44 amino acid C termini found in a variety of Gαi proteins, including Gαt2, Gαi2, and Gαz (Fig. 1*A*) (G16/t2, G16/i2 and G16/z), and assayed them under identical experimental conditions to the experiments of G16/gust chimeras. As a result, G16/t2 and G16/i2 exhibited effective couplings with both mT2R5 and hT2R16 whereas G16/z did not (Fig. 4*A*). The dose-dependent curves were similar to those obtained from G16/gust chimeras that coupled to these receptors (Fig. 4*B*). There are no significant differences in the potency and efficacy between G16/t2 and G16/i2.

Discussion

To determine essential domains for coupling to GPCR, many loss-of-function-type mutation studies on G-proteins have been done. However, mutations of structurally important G-protein domains can also inhibit functional coupling with GPCRs, although the mutation domains themselves are not binding sites for GPCRs. In the present study, we constructed a variety of chimeric Gα proteins to better understand the domains involved in the interactions between T2R and gustducin. In addition, these chimeric Gα proteins enabled us to analyze gustducin-linked GPCRs on common robust assays that are amenable to high throughput-screening analysis. We monitored $[Ca^{2+}]i$ increases caused by activation of signaling cascade T2R-heterotrimeric G-protein (Gαβγ)-phospholipase C-inositol 3,4,5 triphosphate receptor. From the G-protein α, β, and γ subunits, probably both Gα and βγ dimers contact the receptors. The Gα subunit is likely to play a decisive role in discriminating between different receptor subtypes (Savarese and Fraser, 1992; Bourne, 1997; Wess, 1997). Here, we demonstrated that G16/gust44 and G16/gust37 successfully coupled to T2R receptors for their signal transduction. These responses were comparable with those obtained from *in vivo* experiments, and there is evidence that mT2R5 can activate intact gustducin *in vitro* (Chandrashekar et al., 2000), indicating that our system is capable of reproducing signaling transduction of T2R receptors in native TRCs. In contrast, G16/gust23 that contained the α5 helix of gustducin appeared not to associate, although numerous studies have attested to the importance of the α5 helix in receptor coupling. Similarly, G16/gust11 and G16/gust5 did not cause T2R activity. These results indicated that the α5 helix and extreme C terminus of gustducin were insufficient for detection of T2R activities, and the β6 sheet, in addition to the α5 and C-terminal β-sheet, is indispensable for signal transduction of T2Rs.

We next tested whether T2Rs could couple to domains including the β6 sheet, α5, and extreme C terminus from other G-protein α subunits. As a result, we revealed that some G16 chimeras constructed from other Gα proteins, G16/t2 and G16/i2, functionally coupled with the T2R receptors examined. Rod-α-transducin (Gαt1) and cone-α-transducin (Gαt2) are present in vertebrate taste cells. The former has been reported to transduce bitter taste by coupling taste receptor(s) to taste cell phosphodiesterase (Ruiz-Avila et al., 1995). In addition, gustducin and rod-transducin are biochemically indistinguishable in their *in vitro* interactions with retinal phosphodiesterase, rhodopsin (retinal GPCR), and G-portion βγ subunits (Hoon et al., 1995). Because the amino acid sequences of the β6 sheet and α5 helix in Gαt1 and Gαt2 are almost identical to those of gustducin (Fig. 1*A*), the C-terminal region (β6, α5, and extreme C terminus) conserved could be one of the most important domains for α-gustducin, Gαt1, and Gαt2 to interact with GPCRs.

In the present study, functional expression of T2R receptors was also observed in HEK293T cells coexpressing G16/i2 and T2R receptors, suggesting that T2Rs cannot only couple to gustducin and transducin but also to the G-protein αi2 subunit. One or more G-protein α subunits may play a role in bitter taste transduction, because α-gustducin knock-out mice retain residual responsiveness to bitter compound. In addition, transgenic expression of a dominant-negative form of α-gustducin from the gustducin promoter further decreased the residual responses of α-gustducin knock-out mice apparently by inhibiting T2R/TRB interactions with other TRC-expressed G-protein α subunits (Margolskee, 2002). It has been reported that Gαi2 subunit is expressed in subsets of TRCs. The frequency of Gαi2 expression appears to be higher than that of gustducin, and some Gαi2-positive cells also express α-gustducin (Kusakabe et al., 2000). Gαi2 could thus function as "backup" for gustducin in T2R-gustducin-expressing TRCs. In contrast, several TRCs that are immunoreactive for Gαi2 but not for gustducin responded to cycloheximide in an *in vivo* recording using mouse lingual slices (Caicedo et al., 2002). This suggests that Gαi2 may be involved in gustducin-independent bitter taste transduction via other G-protein-coupled receptors as well as T2Rs. On the basis of *in situ* hybridization with a mix of 10 different T2R probes, it was concluded that T2R genes are selectively expressed in gustducin-expressing taste receptor cells (Adler et al., 2000). However, a recent genomic study has shown that mouse T2R (Tas2r) family is composed of at least 36 full-length genes (Shi et al., 2003). Additional studies are required to determine whether all of the T2R genes are exclusively expressed in gustducin-expressing cells.

Many Gαi-coupled GPCRs share the ability to inhibit adenylyl cyclase via the pertussis toxin-insensitive Gαz (Chan et al., 1995; Lai et al., 1995). The incorporation of a Gαz-specific sequence into a Gα16 backbone (G16/z) successfully improved the recog-

nition of a variety of Gαi-coupled receptors (Mody et al., 2000). However, the G16/z chimera was incapable of responding to the T2Rs studied in the present experiments, indicating that T2R receptors have specific sequences for interacting with gustducin and Gαi2. Within the β6 sheet and α5 helix (37 amino acids), there are five amino acids that are conserved in gustducin and Gαi2 but not in Gαz: V333, K346, D350, C351, and F354 in gustducin (at position −23, −10, −5, −4, and −1, respectively; the residues −1 being the last one) (Fig. 1*A*). In particular, the latter three amino acids are contained in the extreme C terminus of the Gα protein. Indeed, a gustducin mutant containing a glycine-to-proline substitution at position −3 can bind to taste receptor Gβγ subunits and the effector, but it cannot be activated by receptors (Ruiz-Avila et al., 2001). Therefore, the extreme C terminus may also play an important role in transduction via the gustducin, Gαt1, Gαt2, and Gαi2 of T2R taste receptors.

In conclusion, we found that 37 C terminal amino acids (β6, α5, and extreme C terminus) of gustducin and Gαi2 are indispensable for the detection of T2R activity. Because T2Rs have the greatest conservation in their cytoplasmic loops and adjacent transmembrane segments, which are the predicted sites for G-protein interaction, the present chimeric G16/gust proteins could be powerful tools to analyze orphan gustducin-linked taste receptors.

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(11) RELATED PROCEEDINGS APPENDIX

None.